Chapter 5

The role of YfiA in ribosomal stalling in *Lactococcus lactis*

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Abstract

Dimerization of ribosomes, so-called ribosome stalling, in*Escherichia coli* is a two-step process. First, ribosome modulation factor RMF holds the large and small subunits of the ribosome together, after which hibernation promotion factor HPF binds to complete the ribosome dimer state. *Lactococcus lactis* MG1363 has a paralog of HPF, annotated as YfiA (YfiA^{Ll}; Llmg0616), but none for RMF. In this study we show that YfiA^{Ll} is involved in the dimerization of ribosomes upon entry of lactococcal cells into the stationary phase. The N-terminal amino acid sequence of YfiA^{Ll} is homologous with that of HPF of *E. coli*. *L. lactis ΔyfiA*, in which the entire gene encoding YfiA has been removed, does not have a strong growth effect, but failed to dimerize ribosomes in the stationary growth phase. Introduction in *L. lactis ΔyfiA* of a variant of YfiA^{Ll} in which the C-terminal 59 amino acids were removed does not restore ribosome dimerization, while complementation with full-length YfiA^{Ll} does. Thus, YfiA^{Ll} is a two-edged sword: the N-terminus of the protein contains the very well conserved HPF domain, while the C-terminus is a functional homolog of the RMF^{Ec} protein.

Introduction

Dimerization of ribosomes in *Escherichia coli* is a stationary phase process that increases the viability of the cell [3]. During the transition phase when one or more nutrients become scarce [4], or when facing stressful conditions [5], *E. coli* 70S ribosomes can form 90S dimers upon binding with ribosome modulation factor RMF [1]. These intermediate ribosome dimers can subsequently bind a hibernation promotion factor (HPF) molecule to form a mature 100S ribosomal particle in which the dimerization interface is located between the two small subunits of the two participating ribosomes [2]. These ribosome dimers represent a hibernation state and are translationally inactive [6]. Ribosomal hibernation has been suggested to constitute a method for cells to prevent ribosomes from being degraded by ribonucleases [7]. A third protein that can bind to ribosomes when *E. coli* cells enter the stationary phase is YfiA (RaiA). HPF and YfiA are structurally similar and by X-ray diffraction and cryo-electron tomography studies it was demonstrated that both proteins can bind to the catalytic A- and P-sites of the ribosome [8,9]. By creating heterologous complexes of *Thermus thermophilus* ribosomes

with *E. coli* YfiA, HPF and/or RMF, detailed information was obtained on the interaction of these proteins with the ribosome and how those lead to ribosome inactivation. RMF blocks the ribosome-binding site of mRNAs, thus preventing interaction of the messenger with the 16S rRNA. HPF and YfiA have nearly identical binding sites in the ribosome, which overlap the sites where the mRNA, tRNA and initiation factors would normally bind. Both HPF and YfiA are in the immediate vicinity of ribosomal proteins S9 and S10 and, because their positively charged surfaces are in close vicinity to conserved residues in the ribosomal peptidyl transferase center, protein translation is disturbed [2].

Multiple alignment and phylogenetic analyses indicate that most bacteria have at least one HPF homologue. These homologues can be classified into three types, long HPF, short HPF and YfiA based on the presence of a conserved domain and additional homologous sequences [10]. An open reading frame annotated as *yfiA* occurs in the genome of the lactic acid bacterium *Lactococcus lactis*. The encoded protein, YfiA^{Ll}, shares 32% amino acid sequence identity with YfiA of *E. coli*. YfiA^{Ll} belongs to the long HPF type: it shares 64% identity with the long HPF sequence of *S. pyogenes* [10]. The *L. lactis* genome does not contain other orthologs of *rmf* and *hpf* [11].

Not much is known about the transcriptional behavior of *yfiA, rmf* and *hpf* genes. It was shown that transcription of *rmf* in *E. coli* is positively regulated by (p)ppGpp [3] and growth rate [5]. *L. lactis yfiA* transcription was shown to be growth rate-dependent (Chapter 2), and transcriptionally activated in the exponential growth phase [12] (Brouwer *et al.,* unpublished).

Here, we studied ribosomal dimerization in *L. lactis* and the role of YfiA therein. Ribosome dimers in *L. lactis* were only seen when full-length lactococcal YfiA was present in the cell. Next to that, we show that the C-terminal tail of YfiA^{Ll} is essential for the formation of ribosomal dimers. A *yfiA* mutant in *L. lactis* was not able to produce ribosome dimers. Finally, with RNA degradation assays we show that dimerization might be beneficial for *L. lactis* by preventing rRNA degradation.

Material and Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* was grown aerobically at 37°C in TY medium (1% Bacto-Tryptone, 0.5% Bacto-yeast extract and 1% NaCl). *L. lactis* strains were grown as standing cultures at 30°C in M17 medium (Difco Laboratories, Detroit, MA) supplemented with 0.5% (w/v) glucose (GM17). Solid media contained 1.5% agar. Chloramphenicol (5 µg/ml) and erythromycin (120 µg/ml for *E. coli* and 2.5 µg/ml for *L. lactis*) were added when required.

General DNA techniques

General molecular biology techniques were performed essentially as described by Sambrook *et al*. [13]. Plasmid DNA was isolated using a High Pure Plasmid Isolation Kit and protocol (Roche Applied Science, Indianapolis, IN). Chromosomal DNA from *L. lactis* was isolated according to the method described by Johansen and Kibenich [14]. Polymerase chain reactions (PCR) were either performed with the Phusion enzyme (Finnzymes, Espoo, Finland) or with a modified version of it, named PfuX7 [15]. This enzyme yields a uracil excision-ready PCR fragment that was subsequently ligated with a mixture of uracil DNA glycosidase and DNA glycosylase-lyase endo VIII, commercially available as USER, using the protocol by the company (New England Biolabs, Ipswich, MA). Colony PCR was performed with the Taq Polymerase (ThermoFisher Scientific Inc, Waltham, MA). Primers listed in Table 2 were purchased from Biolegio BV (Nijmegen, the Netherlands). PCR products were purified with a High Pure PCR Product Purification Kit (Roche Applied Science) according to the protocol of the supplier. DNA electrophoresis was performed in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) in 1% agarose gels with 2 µg/ml ethidium bromide. Electrotransformation was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA). Sequencing reactions were done at MacroGen (Seoul, Korea).

L. lactis YfiA overexpression and protein purification

The *yfiA* gene was amplified by PCR using *L. lactis* MG1363 chromosomal DNA as the template and primers Pr8 and Pr9 (Table 2). The PCR product, consisting of the *yfiA* gene extended at the 5'-end with the codons for the Strep-tag [16], was purified, di-

gested with *Nco*I and *Xba*I and ligated into pNZ8048 [17] cut with the same enzymes. The same strategy was used to clone the *yfiA* gene without the coding region for a Strep-tag (primers Pr7 and Pr9). The resulting plasmids (pNZ*strepyfiA* and pNZ*yfiA*), in which *strepyfiA* and the *yfiA* were under the control of the nisin-inducible promoter P_{nisA}, were obtained in *E. coli* and subsequently introduced in *L. lactis* NZ9000 [18]. Nucleotide sequences were confirmed by nucleotide sequence analysis. YfiA and Strep-tagged YfiA (Strep-YfiA) were overexpressed using the nisin-inducible system (NICE) [17]. Filter-sterilized culture supernatant of the nisin-secreting strain *L. lactis* NZ9700 was used as a source of nisin. Overnight cultures of *L. lactis* NZ9000 carrying either pNZ*strepyfiA* or pNZ*yfiA* were diluted 100-fold in 1 L of fresh GM17 medium with 5 μg/ml chloramphenicol and incubated at 30°C. Nisin-containing supernatant was added (1:500) when the OD_{600} of the culture had reached 0.8. After 4 h of further incubation cells were pelleted (7,000 g for 10 min), resuspended in either 10 ml phosphate-buffered saline (PBS) or in 10 ml PBS with 0.6% paraformaldehyde and incubated at 37°C for 20 min. Cells were centrifuged (7,000 g for 10 min at 4°C), washed once with wash buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.0), resuspended in 10 ml wash buffer and stored at -80°C. Cells were thawed, and treated with 10 mg/ml lysozyme and Complete Mini Protease Inhibitor, according to the manufacturer's instructions (Roche Applied Science) for 60 min at 30°C. Subsequently, 0.1 g DNAseI powder and 10 mM $MgSO_4$ were added and the lysozyme-treated cells were broken using a sonicator for 5 cycles of 45 sec at 65% and 15 sec of incubation on ice (Aminco, Silver Springs, MD). The suspension was centrifuged at 9,000 g (15 min, 4°C). Strep-tagged YfiA was purified to homogeneity using a Streptactin Sepharose column according to the manufacturer's instructions (IBA-GmbH, Göttingen, Germany). Samples from each step in the purification protocol were analyzed by sodium dodecylsulphate 12%-polyacrylamidegel electrophoresis (SDS 12%-PAA) [19] and Western hybridization using anti-Strep-tag antibodies (IBA-GmbH). The concentration of purified protein was determined via spectroscopy (Nanodrop, ThermoFisher Scientific Inc). Protein (100 μM) was kept at -80ºC in 10% glycerol, 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8.0.

SDS-PAA and Western hybridization

Protein samples were mixed (1:1) with sample buffer (120 mM Tris-HCl, pH 6.8, 50% glycerol, 100 mM DTT, 2% (w/v) SDS and 0.02% (w/v) bromophenol blue), vortexed,

boiled for 5 min and separated on SDS 12%-PAA gels. PageRuler Prestained Protein Ladder (ThermoFisher Scientific Inc.) was used as a protein size marker. Gels were stained with Coomassie Brilliant Blue (0.25% Coomassie Brilliant Blue R-250 dissolved in 25% isopropanol with 10% acetic acid) and destained in boiling demineralized water. Western blotting analysis was performed with the SNAP (Millipore Corp., Billerica, MA) system as follows: the PAA gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Proteins were transferred to a PVDF blotting membrane (Roche Applied Science) for 30 min at 20 V. The blot holders containing the blots were placed in the SNAP system, after which blocking buffer in PBST (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.3, 0.1% v/v Tween-20) with 0.5% skim milk was added and the vacuum was applied. Anti-Strep-tag antibodies conjugated to peroxidase (IBA-GmbH), diluted 1000-fold in blocking buffer, were added to the blot holders; incubation was for 10 min at room temperature. The vacuum was removed and the blots were washed three times with PBST prior to visualization of immunoreactive proteins using the ECL detection kit and protocol (GE Healthcare, Buckingham, UK).

Construction of an *L. lactis yfiA* double crossover mutant

Upstream and downstream regions of *yfiA*, PCR-amplified using primer pairs Pr3/Pr4 and Pr5/Pr6 (Table 2), respectively, were inserted in the integration vector pCS1966 [20], amplified with Pr1 and Pr2, and ligated with USER as described above. The resulting plasmid, pCS1966*ΔyfiA*, was obtained in *E. coli* and introduced into *L. lactis* to allow integration via single crossover homologous recombination. An *L. lactis* integrant carrying the pCS1966 construct was selected on GM17 plates with chloramphenicol. Screening for subsequent plasmid excision was done on plates containing 5-fluoroorotic acid by selecting against the *oroP* gene on pCS1966 [20]. A mutant carrying a clean knockout of *yfiA* was obtained and confirmed using PCR and nucleotide sequence analysis.

Complementation of *ΔyfiA*

Complementation of *L. lactis ΔyfiA* was performed with three different plasmids: pIL253*yfiA*^{Ll}, pIL253*yfiA*$^{Ll1\text{-}126}$ and pIL253*yfiA*^{Ec}. The *yfiA* genes from *L. lactis* and *E. coli* were amplified using primer pairs Pr10/Pr11 and Pr12/Pr13, respectively. A truncated version of *L. lactis* YfiA lacking the 59 amino acids at the C-terminus (YfiA$^{Ll1\text{-}126}$) was amplified using primer pair Pr10/Pr14. The amplified products were ligated in the

pIl253 vector [21] amplified with primer pair Pr15/Pr16 employing USER enzyme. Constructs were introduced in *L. lactis* Δ*yfiA* via electrotransformation [22].

Isolation of ribosomes

L. lactis cells were harvested by centrifugation at 7,000 g for 10 min at 4°C after 3 h or 7 h of growth in GM17 at 30°C. The cell pellet was resuspended in buffer I (20 mM Tris-HCl (pH 7.6), 15 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Subsequently, the cells were lysed by mixing them in two cycles with 0.2 mg glass beads in an ice-cold Tissue-lyser (Qiagen, Venlo, the Netherlands). The homogenate was centrifuged at 9,000 g for 15 min at 4°C. The supernatant was saved on ice and the pellet was resuspended in buffer I with 1 mM PMSF. The suspension was centrifuged again under the same conditions. The combined supernatants (cell extracts) were layered onto a 30% sucrose cushion in buffer I and centrifuged in a MLA 80 rotor (Beckman, Fullerton, CA) at 206,000 g for 3 h at 4°C. By resuspending the pellet in buffer I a crude preparation of ribosomes was obtained. To isolate ribosome monomers and dimers, this crude ribosome 400 µl preparation was layered onto a 10–40% 12 ml sucrose density gradient column in buffer I and centrifuged in a SW 32.1 Ti rotor (Beckman) at 125,000 g for 80 min at 4°C [25]. The gradient was fractionated by collecting 400 µl fractions from the top, and the absorbance of each fraction at 260 nm was measured with a UV-1700 spectrometer CARY bio UV-Visible Spectrophotometer (Agilent Technologies, Palo Alto, CA). The fractions containing the ribosome monomers and dimers were dialyzed at 4°C against buffer I and prepared for mass spectrometry, RNA degradation assay and electron microscopy.

Mass spectrometry

For in-solution tryptic digestion, the 400 µl dialyzed fractions containing ribosome monomers and dimers were precipitated overnight at -20ºC using 4 volumes of acetone. The precipitated proteins were first resuspended in 2 µl of 6 M urea and subsequently diluted to 20 µl with 100 mM triethylammonium bicarbonate (TEAB). For reduction and alkylation of the cysteine residues, the samples were incubated for 60 min at 55°C in the presence of 5 mM Tris (2-carboxymethyl) phosphine hydrochloride (TCEP) followed by the addition of 10 mM methyl methanethiosulfo-

nate (MMTS) and incubation at room temperature for 10 min. The protein mixture was incubated overnight with 0.25 µg of trypsin (Trypsin Gold, mass spectrometry grade, Promega 10 ng/µl in 25 mM NH_4HCO_3) at 37 ºC. Subsequently 10 µl of 25 mM NH_4HCO_3 was added to prevent drying, and the incubation was prolonged overnight at 37°C. The tryptic peptides were recovered by three subsequent extractions with 50 µl of 35%, 50%, and 70% acetonitrile in 0.1% TFA. The extracted peptides were pooled and concentrated under vacuum. The digested peptides were analyzed by LC/MSMS on an LTQ Obitrap XL (ThermoFisher) as described previously [26]. In-gel tryptic digestion for mass spectrometry-based protein identification was performed by excision of the gel bands and treated with 10 mM DTT followed by 55 mM iodoacetamide in 50 mM NH_4HCO_3 to reduce and alkylate cysteine residues. Subsequently the gel slices were dehydrated by incubation for 5 min in 100% acetonitrile, rehydrated in 10 µl of trypsin solution (Promega) and incubated for 2 h at 37°C. The digested peptides were analyzed as described above.

RNAse degradation assay

Ribosome fractions from the sucrose gradient were incubated at 30 ºC for 30 min in the presence or absence of 1 µl of 10 mg/ml RNAse (Roche Applied Science), 2 or 3 µl freshly purified Strep-YfiA, and 2 or 3 µl RNAse-free buffer TE-DEPC (Tris-HCl pH 8.0 plus diethylpyrocarbonate) in a total volume of 11 µl. Reaction mixtures in RNA-loading buffer (10 mM Tris-HCl, 10 mM EDTA, 40% v/v glycerol, 0.1% v/v diethylpyrocarbonate) were loaded on an RNA gel [27].

Transfer electron microscopy and single particle analysis

Purified ribosome monomer and dimer samples were prepared for negative staining with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a CM120 electron microscope (Philips, Eindhoven, the Netherlands) equipped with a LaB_6 cathode, operated at 120 kV. Images were recorded with a 4000 SP 4K slow-scan CCD camera (Gatan, Pleasanton, CA) at 80,000-fold magnification with a pixel size of 0.375 nm at the specimen level after binning of the images. GRACE software [28] was used for semi-automated data acquisition. Single particles were analyzed with the Groningen Image Processing software using standard procedures [29]. After multiple alignment steps and multivariate statistical analysis, hierarchical classification based on group averages resulted in several classes, of which

four were visibly distinct and considered representative of the dimer side view. Images were optimized by application of conditional summing with the correlation coefficient of the final alignment step as a quality parameter to select the most homogeneous images in each class (500-2000 images per sum). The final sums were filtered to further reduce noise and bring out smaller details. The surface area of the small and large subunits of both monomer and dimer images were drawn over the final image sums. Subsequently, they were overlaid on the electron micrographs.

Results

YfiA is conserved among Streptococcaceae

Of the proteins involved in ribosome dimerization in *Escherichia coli* [25], ribosome modulation factor (RMF) and hibernation promotion factor (HPF) seem not to be encoded in the genomes of members of the Gram-positive family of Streptococcaceae. Only the gene for a YfiA homolog is present in the annotated streptococcal genomes, and the sequence of this protein is highly conserved (Fig. 1). A member of the Streptococcaceae, *Lactococcus lactis*, contains a single gene (*llmg0616*) coding for the YfiA^{Ll} protein, which is very similar to *Staphylococcus aureus* HPF (SaHPF; Fig. 1). In *E. coli*, RMF and HPF together dimerize ribosomes, while YfiA^{Ec} does not, neither alone nor with any combination of RMF and HPF [1]. In fact, YfiA^{Ec} prevents ribosome dimerization [1]. By contrast, SaHPF was shown to be solely responsible for ribosome dimerization in *S. aureus* [32]. The N-terminus of YfiA^{Ll} has similarities with the *E. coli* proteins YfiA^{Ec} (31%), HPF (32%) (Fig. 1). The similarity is confined to the N-terminus of YfiA^{Ll} because the *E. coli* ribosome dimerization factors are shorter than YfiA^{Ll} and the other streptococcal YfiA proteins, and SaHPF. The latter have a well-conserved C-terminal domain that is not present in the *E. coli* proteins

Growth characteristics of the *L. lactis ΔyfiA* mutant

In order to determine the importance and functionality of *yfiA* for *L. lactis* a clean *yfiA* knockout mutant was made. The fact that it was possible to make *L. lactis ΔyfiA* shows that the gene is not essential. The *yfiA* mutant does not show a growth defect in rich medium, nor is the viability of the culture reduced, as determined by colony counts. The mutation also does not affect the length of the lag-phase after re-inoculation (Fig. 2).

Table 1. Strains and plasmids used in this study.

Strains	Characteristics	Reference
L. lactis NZ9000	MG1363 derivative; plasmid-free, *pepN*::*nisRK*; host for nisin-induced protein expression	18
L. lactis NZ9000 Δ*yfiA*	NZ9000 derivative; chromosomal deletion of *yfiA*	This work
L. lactis NZ9700	Nisin producer strain	30
E.coli MC1061	High efficient transforming strain, host of recombinant plasmids for *L. lactis*	31
Plasmids	**Characteristics**	**Reference**
pCS1966	*oroP*; Em^R; non-replicating, integration vector in *L. lactis*	20
pCS1966 Δ*yfiA*	pCS1966 derivative, carrying flanking regions of *yfiA*	This work
pNZ8048	Cm; nisin inducible expression vector carrying P_{nisA}; *NcoI* site used for translational fusions	17
pNZ*strepyfiA*	pNZ8048 derivative, with *yfiA* containing an extra coding region for a N-terminal Strep-tag inserted under the *nisA* promoter	This work
pIL253	Em^R; High-copy number inducible expression vector carrying P_{ara} and *lac* genes for selection	21
pIL253 $yfiA^{Ll}$	pIL253 derivative, with *yfiA* of *L. lactis*	This work
pIL253 $yfiA^{Ll1\text{-}126}$	pIL253 derivative, with *yfiA* of *L. lactis,* truncated 59 amino acids at the C-terminus	This work
pIL253 $yfiA^{Ec}$	pIL253 derivative, with *yfiA* of *E. coli* K12	This work

Table 2. Primers used in this study. Restriction sites are underlined. For Pr8, the nucleotides encoding the Strep-tag are in bold and those representing the linker between the *strep*-tag and *yfiA* are in italic.

Primer name	**Sequence** (5' - 3')	**Purpose**
Pr1	ATCGTACCCUCGAGTGTTCCCTTTAGT-GAGGGT	Amplification of pCS1966 vector
Pr2	ATACTAGTTCUAGAGCGGCCGCCAACAACC	Amplification of pCS1966 vector
Pr3	AGAACTAGTAUGACTAAATCTGAAAGCGAC-CG	Amplification of *yfia* up-stream region
Pr4	ATCCTTTGAUCATAAGAGTACCTCTTC	Amplification of *yfia* up-stream region
Pr5	ATCAAAGGAUCCACAGAATAAAAATTAAGG	Amplification of *yfia* downstream region
Pr6	AGGGTACGAUCATGAATTCTTGGAAAGC	Amplification of *yfia* downstream region
Pr7	GGCCGCCATGGTCAAATTTAATATCCGTGG	Placing *yfia* downstream of P_{nisA}
Pr8	GGCCGCCATGGCT**TGGAGCCAT CCCAATTTGAAAAA***GGT TCTAAAAGC*ATCAAATTTAATATCCG	Adding an N-terminal Strep-tag to *yfia*
Pr9	GGCCGTCTAGATAATTTTTATTCTGTTTC	Placing *yfia* downstream of P_{nisA}
Pr10	ATTTTGCAUGATCAAATTTAATATCCGTGGC-GAA	Amplification of *L. lactis yfiA* gene
Pr11	ACTTGAUTTATTATTCTGTTTCAATTAAGC-CATAACGACCATCTG	Amplification of *L. lactis yfiA* gene
Pr12	ATTTTGCAUGACAATGAACATTACCAGCAAAC	Amplification of *E. coli yfiA* gene
Pr13	ACTTGAUCTACTCTTCTTCAACTTCTTCGAC	Amplification of *E. coli yfiA* gene
Pr14	ACTTGAUTTATTAATCCTCAGCAACTTCAT-CAG	Amplification of *L. lactis yfiA* gene, removing 59 amino acids from C-terminus
Pr15	ATCAAGUGTTCGCTTCGCTCTCACTG	Amplification of pIL253 vector
Pr16	ATGCAAAAUTCCTCCGAATATTTTTT-TACCTACC	Amplification of pIL253 vector

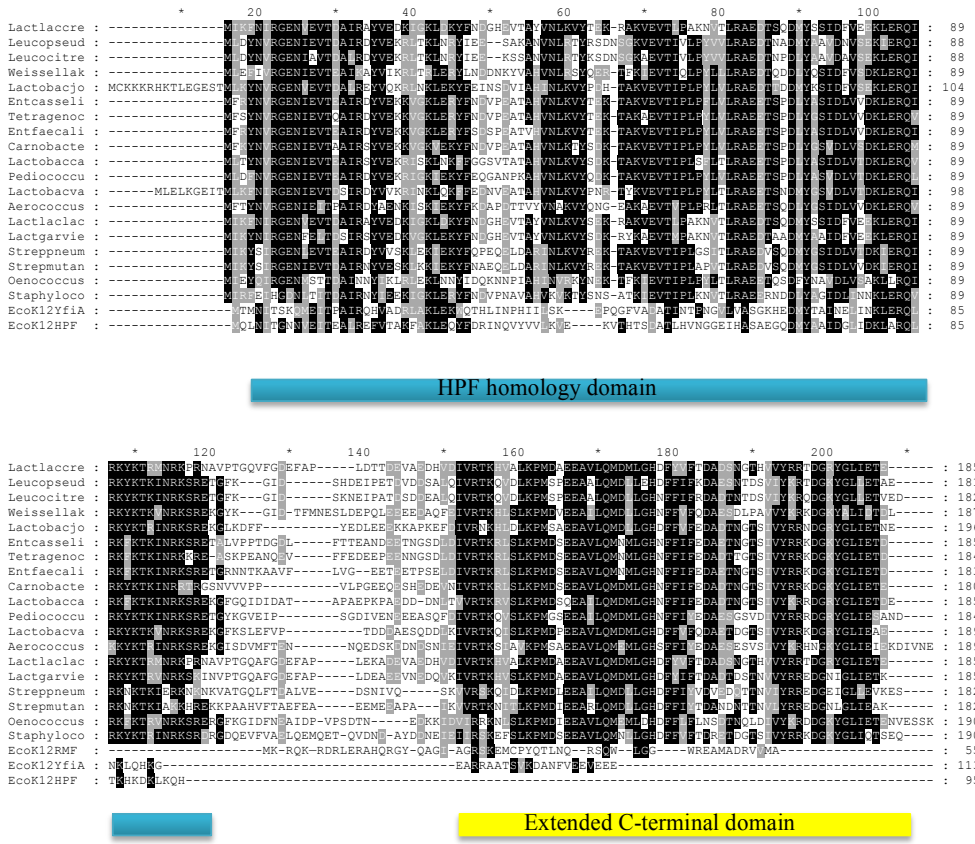

Figure 1. Sequence alignment of YfiA of streptococcal members and compared with *E. coli* YfiA HPF and RMF. The multiple sequences were aligned with ClustalW [31] and presented using Boxshade 3.2.3 [30]. Black backgrounds indicate identical residues and grey backgrounds indicate conserved residues. The HPF homolog conserved domain (blue box) and the extended C-terminal domain (yellow box) are adapted from Ueta *et al.,* 2008 [10]. Leucopseud: *Leuconostoc pseudomesenteroides*, Leucocitre: *Leuconostoc citreum* KM20, Weissellak: *Weissella koreensis* KACC15510, Lactobacjo: *Lactobacillus johnsonii* ATCC3320, Entcasseli: *Enterococcus casseliflavus* EC10, Tetragenoc: *Tetragenococcus halophilus* NBRC1, Entfaecali: *Enterococcus faecalis* V583,Carnobacte: *Carnobacterium sp.* AT7, Lactobacca: *Lactobacillus casei* ATCC334, Pediococcu: *Pediococcus pentosaceus* ATCC2574, Lactobacva: *Lactobacillus vaginalis* ATCC4954, Aerococcus: *Aerococcus urinae* ACS-120-V-Col1, Lactlaccre: *Lactococcus lactis* ssp. *cremoris* MG1363, Lactlaclac: *Lactococcus lactis* ssp. *lactis* Il1403, Lactgarvie: *Lactococcus garvieae* ATCC49156, Streppneum: *Streptococcus pneumoniae* TIGR4, Strepmutan: *Streptococcus mutans* UA159, Oenococcus: *Oenococcus oeni* PSU-1, Staphyloco: *Staphylococcus aureus* ssp. *aureus,* EcoK12RMF: *Escherichia coli* K12RMF EcoK12YfiA: *Escherichia coli* K12, EcoK12HPF: *Escherichia coli* K12HPF.

Ribosome dimerization in *L. lactis*

Subsequently, we examined whether *L. lactis* ribosomes dimerize during stationary phase, and whether or not this was abrogated in *L. lactis ΔyfiA*. The dimerization state of ribosomes in both strains was investigated using sucrose-gradient ultracentrifugation. Using this procedure, ribosomes sediment at a specific sucrose percentage according to their sedimentation or Svedberg (S) coefficient. Cells of the wild-type strain, harvested in the logarithmic phase of growth, showed a single major peak. Below, we show that the fractions constituting this peak contain 70S ribosomes (Fig. 3A). Dimerization of ribosomes, indicated by the formation of particles sedimenting at 100S, exclusively occurs in the wild-type cells that were harvested in the stationary phase (Fig. 3B). Not all ribosomes dimerize or a fraction may dissociate in the sample preparation, as a minor fraction of 70S ribosomes still remains. In cells lacking $YfiA^{Ll}$, 100S ribosome particles are never observed, neither in exponentially growing (data not shown) nor in stationary phase cells (Fig. 3C).

To verify the constitution and conformation of the ribosomes in the 70S and 100S peaks, fractions were taken and subjected to electron microscopy. Indeed, 70S fractions contain monomeric ribosomes (Fig. 3GI), while in the 100S fractions the ribosome dimers are present (Fig. 3HJ). After aligning and filtering of the electron microscopy images, at least 500 homologous images per view were selected and summed to obtain presented images (Fig. 3G-K). Ribosomes that dimerize are present in a specific conformation with the dimer interface located between the small subunits of two intact ribosomes (Fig. 3H). As is clear from the superpositions presented in Fig. 3IJK, the relative position of the small and large subunits of the ribosome in the dimerized state has changed relative to that of the monomeric ribosome.

The C-terminus of $YfiA^{Ll}$ is essential for ribosome dimerization

As presented above, dimerization of ribosomes does not occur in *L. lactis ΔyfiA* (Fig. 3C). Ribosome dimerization was restored by complementing the mutation in this strain with plasmid pIL*yfiA*^{Ll}, in which *yfiA* was (over)expressed by induction with nisin of P_{nisA}::*yfiA*^{Ll} (Fig. 3D). A C-terminally truncated variant of the $YfiA^{Ll}$ protein lacking the 59 C-terminal amino acids, $YfiA^{Ll1\text{-}126}$, did not restore the ribosome dimerization defect in *L. lactis ΔyfiA* (Fig. 3E). Full-length $YfiA^{Ec}$ is shorter than $YfiA^{Ll}$. It is homologous to the N-terminal domain of $YfiA^{Ll}$ but lacks the conserved

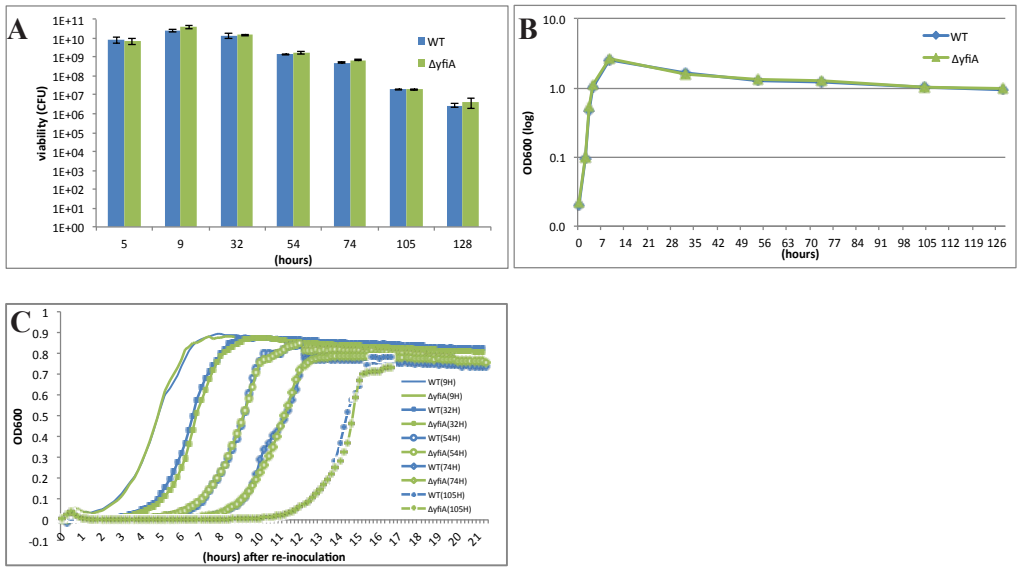

Figure 2. Viability of *L. lactis* wild-type (WT) and Δ*yfiA* strains. (A) Viability in GM17 medium. Samples were taken at the indicated time-points and used to re-inoculate GM17 (1:100) to measure the subsequent lag-phases (B) and plated to GM17 plates to determine cell viability (C). The legenda of C indicates after how many hours re-inoculation was performed. Results are presented as average of triplicate experiments.

C-terminal domain of YfiA^{Ll} (see above and Fig. 1). Overexpressing *E. coli yfiA* in *L. lactis ΔyfiA* does not lead to dimerization of lactococcal ribosomes (Fig. 3F).

YfiA is present in ribosome dimers of *L. lactis*

Ribosome monomers (70S fraction) and dimers (100S fraction) from the sucrose gradients (Fig. 3A-F) were subjected to mass spectrometric analysis. As expected, ribosomal proteins were detected in both samples. Ribosomal proteins were by far the most abundant in the ribosome monomer fraction and other proteins detected were very low in abundance and probably contaminants. Additional proteins that were exclusively present in the ribosome dimer preparations were the ribosomal protein RplL (L7/L12) and the pyruvate dehydrogenase components PdhB and PdhC. The fourth protein in the dimer fraction was YfiA^{Ll} (Fig. 4A).

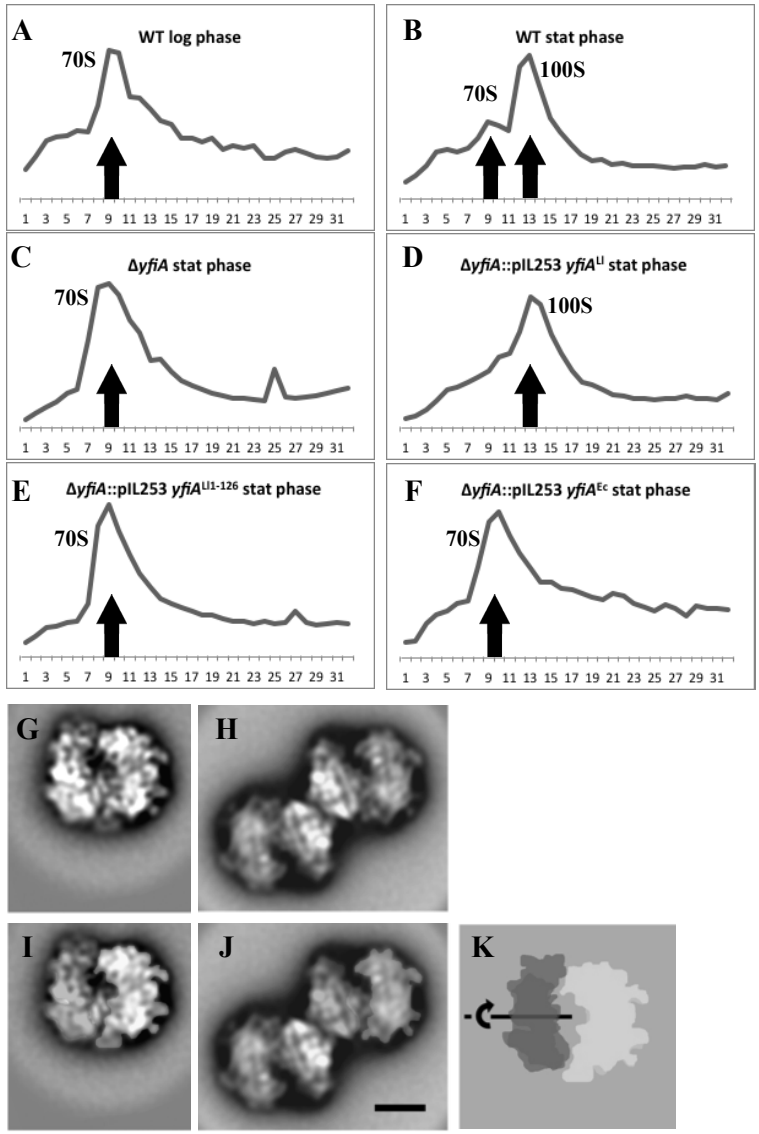

Figure 3. Ribosome formation in *L. lactis*. Ribosome profiles of *L. lactis* after 5-25% sucrose gradient ultracentrifugation were obtained (A-F). The x-axis shows the fraction numbers (top to bottom), the peak heights are derived from the absorbance at 260 nm. Arrows indicate the fractions used for mass spectrometry, RNA degradation assay and electron microscopy. Cells were grown in GM17 at 30°C and were harvested in the logarithmic growth phase (log phase) or in the stationary phase (stat phase). Peak-containing fractions number 9 and 13 were subjected to electron microscopy, where the presence of 70S monomer (G and I) and 100S dimer (H and J) was confirmed. By superpositioning the large (yellow) and small (purple or pink) subunits, a tordation of the small subunits relative to the large subunit between the monomer (I) and the dimer (J) can be observed (K).

Protein interactions of *L. lactis* YfiA

YfiA containing an N-terminal Strep-tag (Strep-YfiA; 22.8 kDa) was overexpressed in *L. lactis* grown to stationary phase and purified by Strep-Tactin purification. We chose to place the Strep-tag on the N-terminus, since the C-terminus appeared to play a major role in the dimerization, giving a risk of diminishing functionality when hooking it up with a Strep-tag. A protein of the expected size (approximately 26 kDa) was visible in a Coomassie-stained SDS 12%-PAA gel and on a Western blot after immunolabeling with anti-Strep antibodies (Fig. 4B). A second protein with a size of approximately 45 kDa also reacts with the anti-Strep antibodies. When the cells were treated with 4% paraformaldehyde (PFA) prior to harvesting and subsequent affinity purification, a smear of proteins with sizes ranging from 50 kDa to 150 kDa was detected by Coomassie staining and Western hybridization (Fig. 4B). The lower amount of Strep-YfiA observable after PFA-fixation and the appearance of a smear of bands at positions of high-molecular weight proteins in the Western blot indicate that Strep-YfiA can form complexes with other proteins. When the experiment was performed without PFA-fixation no clear high-molecular weight products were observed (Fig. 4B). The proteins from the fixated sample and the material in the equivalent gel area from the non-fixated sample were eluted from equally sized gel-slices and subjected to MS/MS. As a control, a similar area of an SDS 12%-PAA gel, containing the proteins from stationary-phase cells from the *L. lactis* wild-type strain, was also examined (Table 3). Table 3 shows a selection of proteins that were identified by MS/MS that appear to have an interaction with Strep-YfiA. In the non-fixated sample, a number of proteins appeared on the gel around the molecular weight of the specific protein plus approximately 22.8 kDa for Strep-YfiA (Table 3), suggesting that direct interactions might exist between Strep-YfiA and these proteins. A number of these proteins are (possibly) involved in protein synthesis (e.g. EF-Tu, RpsA, EF-G and IF-2). We also observed co-purification of proteins involved in central metabolism, like PdhC and PdhD. These proteins, part of the pyruvate dehydrogenase complex, are known in bacteria to co-purify with rRNA modification enzymes as well as with 50S ribosome subunits [33,34]. Also, the PDH-complex is protected against trypsin digestion by membrane-bound ribosomes [36]. Other ribosomal proteins and glycolytic enzymes were not observed in these high molecular weight fractions (Table 3). The only protein not belonging to either of these two groups is the cell division protein FtsZ. Cross-linking with PFA leads to very large complexes. All of the aforementioned proteins that were pulled down

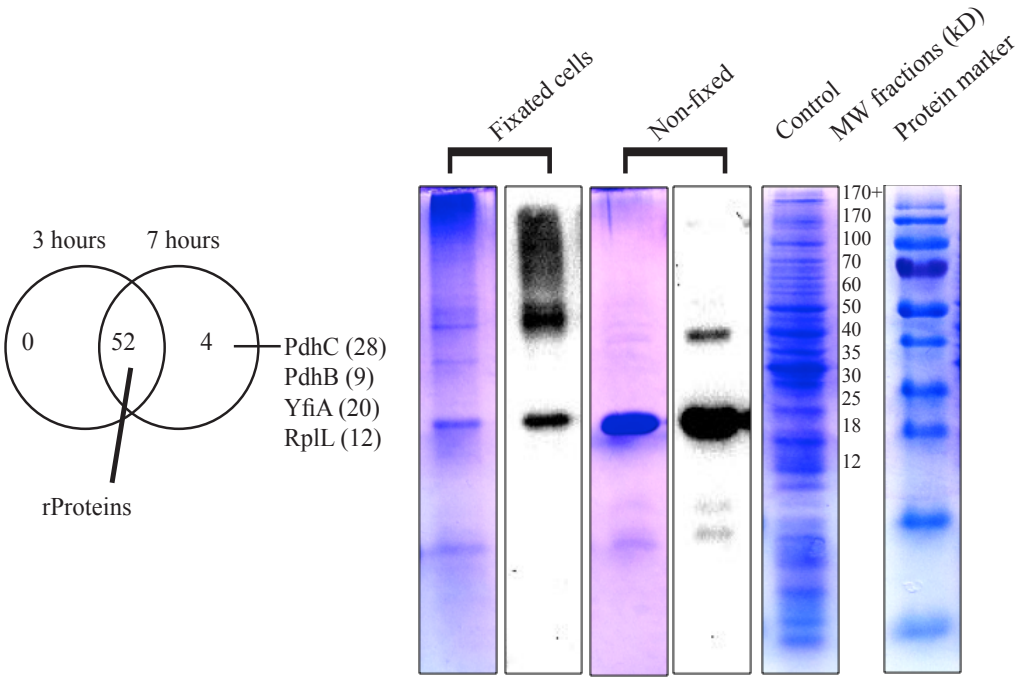

Figure 4. Identification of binding partners of YfiA. (A) After fixation with 4% PFA or not, cells (*L. lactis* overexpressing Strep-tagged YfiA) grown for 7 h in GM17 at 30°C were harvested and disrupted. The protein extracts were subjected to SDS-12% PAA and Coomassie staining (panels 1,3 and 5, from the same gel) and Western hybridization using anti-Strep-tag antibodies (panels 2 and 4, from one blot from a duplicate gel). After that, gels were cut in fractions, based on their molecular weight as indicated by the numbers on the right and proteins were identified by MS/MS. See Table 1 for the numbering system. (B) Proteins identified in ribosome fractions after sucrose gradient ultra-centrifugation of *L. lactis* wild-type cells after 3 h of growth in GM17 at 30°C (logarithmic growth phase) and 7 hours of growth (stationary growth phase). 52 proteins were found with high significance in both samples, and these are mainly ribosomal proteins. In the stationary phase fraction 4 unique proteins were identified, namely YfiA, PdhC, PdhB and RplL (L7/L12), the numbers behind the proteins indicate the spectral counts.

with Strep-YfiA in the absence of PFA appear in these complexes after cross-linking, which can be up to and exceeding 170 kDa.

YfiA prevents ribosomal degradation

Previous studies have shown that stabilization of the 50S/30S conformation of intact ribosomes with Mg^{2+} prevents their degradation by ribonuclease [7]. We investigated whether ribosomes from *L. lactis* are protected against ribonuclease activity when purified Strep-YfiA is added to the reaction mixture. As can be seen in Fig. 5, elec-

Table 3. Proteins that co-purify with Strep-YfiA^{LI}. Nisin-induced *L. lactis* (pNZ*strepyfiA*), grown to stationary phase, were treated with 4% PFA (fixated), or not (non-fixated). The cells were disrupted and the proteins were subjected to Strep-tactin column chromatography. Bound proteins were specifically eluted with desthiobiotin and separated by SDS 12%-PAGE (Fig. 4B). The gel was sliced into 12 equally-sized slices containing proteins of up to 12 kDa and around 18, 25, 30, 35, 40, 50, 60, 70, 100, 170 or larger than 170 kDa. As a control, proteins from L. lactis wild-type stationary phase cells were separated on the same SDS 12%-PAA gel. Proteins were eluted and subjected to MS/MS analysis in which the proteomes of the various samples were determined. Only co-purified proteins are shown here. A protein was identified to co-purify with Strep-YfiA^{LI} when it was detected in a gel slice from the non-fixated cells that contained proteins at least appr 20 kDa larger than the native molecular mass of that specific protein and not in the equivalent gel slice from the control cells.

Identified protein	Annotation	Mass (kDa)	Control cells, occurrence in gel slice	Non-fixated cells, occurrence in gel slice	Fixated cells, occurrence in gel slice
YfiA	Putative sigma 54 protein	21	18	All gel slices	All gel slices
RpsB (S2)	Ribosomal protein S2	29	25; 30	40	100; 170; 170+
RpoA	RNA polymerase subunit α	34	35	50	170+
GapB	Glyceraldehyde 3-phosphate dehydrogenase	36	35; 40	60	70; 100; 170; 170+
Pfk	6-phosphofructokinase	36	30	50	170; 170+
Tsf	Elongation factor Ts	37	35	50	170+
CcpA	Catabolite control protein	37	40	50	170; 170+
TufA	Elongation factor Tu	43	40	60	100; 170; 170+
FtsZ	Cell division protein	44	40	60	170+
RpsA (S1)	Ribosomal protein S1	45	40	60	100; 170; 170+
Tig	Trigger factor	47	50	70	170+
PdhD	Dihydrolipoamide dehydrogenase	50	50	70	170+
PdhC	Pyruvate dehydrogenase component	56	50; 60	100	170+
GroEL	Chaperonin	57	60	70	170+
DnaK	Chaperone protein	65	60	100	170+
GlmS	D-fructose-6-phosphate amidotransferase	66	50; 60	100	170+
TypA	GTP-binding protein	68	60	100	170+
FusA	Elongation factor G	78	70	100	170; 170+
PurL	Phosphoribosylformylglycinamidine synthase II	80	60; 70; 100	100	170+
GyrA	Gyrase subunit A	93	70	170	170+
AlaS	Alanyl-tRNA synthase	96	70	170	170+
ClpB	ATP-dependent protease	97	70	100; 170	170+

AdhE	Alcohol-acetaldehyde dehydrogenase	98	70; 100	170	170+
InfB	Translation initiation factor IF-2	105	100	170	170+

trophoresis of the 70S ribosomal fraction leads to three bands on a standard RNA gel. The upper fragment seems protected, but the smaller subunits 23S and 16S were not, against degradation after incubation with Strep-YfiA. The same ribosomal fractions without Strep-YfiA were completely degraded within 30 minutes of incubation with RNAse (Fig. 5). Nuclease protection by Strep-YfiA is specific for ribosomal RNA, since mRNA degradation could not be prevented (data not shown). It is tempting to speculate that the upper band represents the 70S ribosome, representing an on-gel disrupted ribosomal dimer and the lower bands the monomeric subunits, although this should be verified by Northern blots.

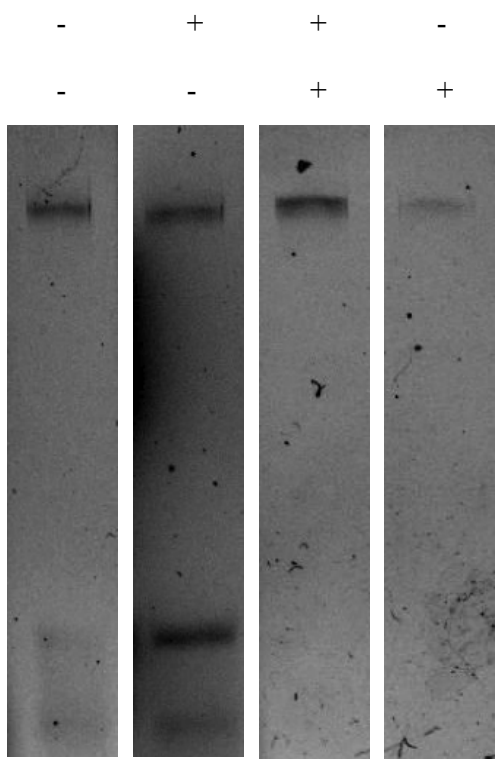

Figure 5. *In vitro* RNA degradation assay. Isolated ribosome fractions were loaded on an RNA gel and incubated with Strep-YfiA or TE-DEPC or RNAse.

Discussion

Here, we identify the protein YfiA^{Ll} as an essential factor in ribosome dimerization in *L. lactis*. Dimerization of ribosomes in this organism takes place when the cells enter into stationary phase. YfiA^{Ll} is not essential as cells lacking the *yfiA* gene are viable. Since the *yfiA* mutant does not show ribosome dimers, it can be concluded that other proteins, such as putative functional homologs of the *E. coli* dimerization proteins HPR and RMF, cannot take over that function in *L. lactis*. Heterologously overexpressed YfiA^{Ec} from *E. coli* did not rescue the *yfiA* mutation in *L. lactis*. Also, C-terminally truncated YfiA$^{Ll1\text{-}126}$ does not allow ribosome dimer formation. Thus, important dimerization functions in YfiA^{Ll} are located in the C-terminus of the protein.

L. lactis YfiA is an example of the so-called long HPF type [10]. In that respect, it resembles the SaHPF protein from *S. aureus*. This protein has been shown recently to be involved in ribosome dimerization [32]. Apart from the domain that is conserved in all HPF homologs, both proteins contain an extended, long and mutually similar C-terminal domain [10]. YfiA^{Ec} contains only a short C-terminal extension to its conserved HPF-domain (Fig. 1). In *E. coli*, this extension of YfiA^{Ec} prevents ribosome dimerization by interfering with RMF-dependent 90S formation. In the present study we show that the extended C-terminal domain in YfiA^{Ll} performs a completely opposite function. It is crucial for dimerization of ribosomes in *L. lactis* during stationary phase. The C-terminus of YfiA^{Ll} functionally resembles *E. coli* RMF, as suggested previously for that of SaHPF [32]. Indeed the C-terminal domains of SaHPF and YfiA^{Ll} show limited homology (Fig. 1).

In a follow-up experiment, *L. lactis ΔyfiA* will be complemented with YfiA$^{Ll1\text{-}126}$ in combination with a protein fragment encompassing the C-terminal domain of YfiA^{Ll} or *E. coli* RMF. This should prove whether the C-terminal domain of YfiA^{Ll} indeed is a functional homolog of RMF^{Ec}. The amino acid residues at the extreme end of the YfiA$^{Ll1\text{-}126}$ protein could interfere with RMF^{Ec} binding, as was postulated on the basis of the model for YfiA^{Ec} binding to the ribosome.

The relative position of the large 50S and small 30S subunits in the lactococcal ribosome monomers has changed in the dimers compared to the monomers (Fig. 3G-K). In *E. coli* it has been shown that RMF^{Ec} is involved in repositioning of the 30S subunit relative to the 50S subunit. The hypothesis is that this conformational change

facilitates the formation of 100S ribosome dimers [2]. Similarly, we propose that the C-terminus of YfiA^{Ll} performs the RMF^{Ec}-like function of inducing the conformational change, allowing ribosome dimers to be formed in *L. lactis*.

L. lactis ΔyfiA does not have a strong phenotype. The growth-rates of the mutant and the wild-type strain do not show differences. Viability and re-growth were also equal for both strains. A minor phenotype was reported for *E. coli yfiA*::*Km*. The cells of this strain may live somewhat longer in stationary phase [1]. The enhanced viability has been explained by increased protection of 70S ribosomes and ribosome dimers against degradation by RNA hydrolases [7,9]. Ribosome dimerization is thought to allow rapid recovery of translation by tmRNA, an RNA molecule with tRNA- and mRNA-mimicking domains. tmRNA is responsible for *trans*-translation, which takes place when ribosomes are stalled on, for instance, damaged mRNA [37]. Interestingly, parts of the tmRNA complex were found in this study to co-purify with Strep-YfiA^{Ll} in stationary phase cells. Of the proteins known to be part of the tmRNA complex, EF-Tu [38] and ribosomal protein S1 [39] were indeed detected. The third and essential protein factor for *trans*-translation, SmpB [40,41] was not detected. This is most likely because this protein does not directly interact with Strep-YfiA^{Ll}, but we cannot exclude that trypsin-digested SmpB is too small to allow detection by our assay. Whether and how YfiA^{Ll} plays a role in *trans*-translation is not known, and needs further research.

Other factors known to interact with the ribosome and involved in various aspects of translation and ribosome recycling were co-purified with Strep-YfiA^{Ll}. We identified initiation factor 2 (InfB), a protein that allows fMet-tRNA and 30S and 50S subunits to form a 70S ribosome [42] and elongation factor Ts, catalyzing the release of guanosine diphosphate from EF-Tu. Also, protein-folding factors such as DnaK, GroEL and trigger factor TG were co-purified. Co-purification with Strep-YfiA^{Ll} of glycolytic enzymes could be the result of unspecific binding since these proteins are very abundant in the cytosol [43]. Also in *S. aureus*, glycolytic enzymes were shown to sediment together with ribosomes after a modified 2D-PAGE separation [32]. More specifically, in that study and in ours, proteins of the pyruvate dehydrogenase (PDH) complex were identified. This co-purification of (parts of) the PDH complex with YfiA^{Ll} need not be an artifact. It has been shown previously that the so-called S-complex in *B. subtilis,* or a membrane-bound ribosome protein complex in *S. aureus*, in fact consists of a membrane-located complex of 4 pyruvate dehydrogenase proteins in tight contact with ribosomes. In fact, the ribosomes were shown to protect the PDH complex from being degraded by trypsin [36]. It is an intriguing possibility that, conversely, the ribo-

some dimers are protected from RNAses by their location at or near the membrane-located PDH complex. The complex would thus serve as a “parking lot” for hibernated ribosome dimers, a speculation that warrants further research.

References

1. Ueta, M. *et al.* Ribosome binding proteins YhbH and YfiA have opposite functions during 100S formation in the stationary phase of *Escherichia coli*. *Genes Cells* **10**, 1103–1112 (2005).
2. Polikanov, Y. S., Blaha, G. M. & Steitz, T. A. How hibernation factors RMF, HPF, and YfiA turn off protein synthesis. *Science* **336**, 915–918 (2012).
3. Yamagishi, M. *et al.* Regulation of the *Escherichia coli rmf* gene encoding the ribosome modulation factor: growth phase- and growth rate-dependent control. *EMBO J.* **12**, 625–630 (1993).
4. Roche, E. D. & Sauer, R. T. SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. *EMBO J.* **18**, 4579–4589 (1999).
5. Izutsu, K. *et al. Escherichia coli* ribosome-associated protein SsrA, whose copy number increases during stationary phase. *J. Bacteriol.* **183**, 2765 –2773 (2001).
6. Wada, A., Igarashi, K., Yoshimura, S., Aimoto, S. & Ishihama, A. Ribosome modulation factor: stationary growth phase-specific inhibitor of ribosome functions from *Escherichia coli*. *Biochem. and Biophys. Res. Comm.* **214**, 410–417 (1995).
7. Zundel, M. A., Basturea, G. N. & Deutscher, M. P. Initiation of ribosome degradation during starvation in *Escherichia coli*. *RNA* **15**, 977 –983 (2009).
8. Vila-Sanjurjo, A., Schuwirth, B.-S., Hau, C. W. & Cate, J. H. D. Structural basis for the control of translation initiation during stress. *Nat. Struct. Mol. Biol.* **11**, 1054–1059 (2004).
9. Ortiz, J. O. *et al.* Structure of hibernating ribosomes studied by cryoelectron tomography *in vitro* and *in situ*. *J. Cell Biol.* **190**, 613 –621 (2010).
10. Ueta, M. *et al.* Role of HPF (Hibernation Promoting Factor) in translational activity in *Escherichia coli*. *J. Biochem.* **143**, 425 –433 (2008).
11. Wegmann, U. *et al.* Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. *J. Bacteriol.* **189**, 3256–3270 (2007).
12. De Jong, A., Ejby, M., Kuipers, O. P., Kilstrup, M. & Kok, J. Detection of environmental changes by *Lactococcus lactis* MG1363 during fermentation of milk. *PLoS ONE* (2013).
13. Sambrook, J., Fritsch, E. F. & Maniatis, T. Molecular cloning: a laboratory manual. (1989).
14. Johansen, E. & Kibenich, A. Isolation and characterization of IS1165, an insertion sequence of *Leuconostoc mesenteroides* subsp. *cremoris* and other lactic acid bacteria. *Plasmid* **27**, 200–206 (1992).
15. Nørholm, M. H. A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. *BMC Biotechnol.* **10**, 21 (2010).
16. Skerra, A. & Schmidt, T. G. Use of the Strep-Tag and streptavidin for detection and purification of recombinant proteins. *Meth. Enzymol.* **326**, 271–304 (2000).
17. De Ruyter, P. G., Kuipers, O. P. & De Vos, W. M. Controlled gene expression

systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl. Environ. Microbiol.* **62**, 3662–3667 (1996).
18. Kuipers, O. P., De Ruyter, P. G. G., Kleerebezem, M. & De Vos, W. M. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* **64**, 15–21 (1998).
19. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970).
20. Solem, C., Defoor, E., Jensen, P. R. & Martinussen, J. Plasmid pCS1966, a new selection/counterselection tool for lactic acid bacterium strain construction based on the orop gene, encoding an orotate transporter from *Lactococcus lactis*. *Appl. Environ. Microbiol.* **74**, 4772 –4775 (2008).
21. Simon, D. & Chopin, A. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. *Biochimie* **70**, 559–566 (1988).
22. Holo, H. & Nes, I. F. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* **55**, 3119–3123 (1989).
23. Hakenbeck, R., Chhatwal, S., Prudhomme, M. & Claverys, J.-P. Molec. Biol. of Streptococci. (2007).
24. Pinto, J. P. C. *et al.* pSEUDO, a genetic integration standard for *Lactococcus lactis*. *Appl. Environ. Microbiol.* **77**, 6687–6690 (2011).
25. Maki, Y., Yoshida, H. & Wada, A. Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. *Genes Cells* **5**, 965–974 (2000).
26. Drop, B. *et al.* Photosystem I of *Chlamydomonas reinhardtii* contains nine light-harvesting complexes (Lhca) located on one side of the core. *J. Biol. Chem.* **286**, 44878–44887 (2011).
27. Aranda, P. S., LaJoie, D. M. & Jorcyk, C. L. Bleach gel: A simple agarose gel for analyzing RNA quality. *Electrophoresis* **33**, 366–369 (2012).
28. Oostergetel G.T., Keegstra W. & Brisson A. Automation of specimen selection and data acquisition for protein electron crystallography. *Ultramicroscopy* **74**, 47–59 (1998).
29. Boekema, E. J., Van Roon, H., Van Breemen, J. F. & Dekker, J. P. Supramolecular organization of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes. *Eur. J. Biochem.* **266**, 444–452 (1999).
30. Rauch, P. J. & De Vos, W. M. Characterization of the novel nisin-sucrose conjugative transposon Tn5276 and its insertion in *Lactococcus lactis*. *J. Bacteriol.* **174**, 1280 –1287 (1992).
31. Casadaban, M. J. & Cohen, S. N. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Molec. Biol.* **138**, 179–207 (1980).
32. Ueta, M., Wada, C. & Wada, A. Formation of 100S ribosomes in *Staphylococcus aureus* by the hibernation promoting factor homolog SaHPF. *Genes Cells* **15**, 43–58 (2010).

33. Jiang, M. *et al.* The *Escherichia coli* GTPase CgtAE is involved in late steps of large ribosome assembly. *J. Bacteriol.* **188**, 6757–6770 (2006).
34. Sergiev, P. V. *et al.* How much can we learn about the function of bacterial rRNA modification by mining large-scale experimental datasets? *Nucleic Acids Res.* (2012).
35. Hemilä, H., Palva, A., Paulin, L., Arvidson, S. & Palva, I. Secretory S complex of *Bacillus subtilis*: sequence analysis and identity to pyruvate dehydrogenase. *J. Bacteriol.* **172**, 5052–5063 (1990).
36. Adler, L. A. & Arvidson, S. Correlation between the rate of exoprotein synthesis and the amount of the multiprotein complex on membrane-bound ribosomes (MBRP-complex) in *Staphylococcus aureus*. *J. Gen. Microbiol.* **133**, 803–813 (1987).
37. Keiler, K. C. Biology of *trans*-translation. *Annu. Rev. Microbiol.* **62**, 133–151 (2008).
38. Rudinger-Thirion, J., Giegé, R. & Felden, B. Aminoacylated tmRNA from *Escherichia coli* interacts with prokaryotic elongation factor Tu. *RNA* **5**, 989–992 (1999).
39. Ramrath, D. J. F. *et al.* The complex of tmRNA-SmpB and EF-G on translocating ribosomes. *Nature* **485**, 526–529 (2012).
40. Karzai, A. W., Susskind, M. M. & Sauer, R. T. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J.* **18**, 3793–3799 (1999).
41. Weis, F. *et al.* tmRNA-SmpB: a journey to the centre of the bacterial ribosome. *EMBO J.* **29**, 3810–3818 (2010).
42. Myasnikov, A. G., Simonetti, A., Marzi, S. & Klaholz, B. P. Structure–function insights into prokaryotic and eukaryotic translation initiation. *Curr. Opin. Struc. Biol.* **19**, 300–309 (2009).
43. Steen, A., Wiederhold, E., Gandhi, T., Breitling, R. & Slotboom, D. J. Physiological adaptation of the bacterium *Lactococcus lactis* in response to the production of human CFTR. *Molec. Cell. Proteomics* **10**, (2011).